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DNA Probes for the Identification of *Nocardia asteroides*

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DNA probes for the rapid identification of *Nocardia asteroides* were obtained by constructing a genomic library of strain GUH-2 in the lambda cloning vector EMBL3. Of 50 recombinant clones tested, 2 were identified that hybridized with 31% of the *N. asteroides* strains in a reference collection without cross-hybridization with related members of the *Actinomycetales*. Additional libraries were then generated from selected strains of *N. asteroides* that had failed to hybridize with any of the GUH-2 clones. Four additional clones were obtained from these strains which, when pooled, provided DNA probes specific for all of the *N. asteroides* strains tested.

The *Nocardia* species most often encountered in human infections are *N. asteroides*, *N. brasiliensis*, and *N. otitidiscaviarum*. The clinical findings commonly associated with these organisms are nocardial actinomycetoma and pulmonary and systemic nocardiosis. *N. asteroides* is the most common cause of pulmonary nocardiosis and, with its tendency to disseminate to other organs, offers a poor prognosis for the patient (3). Early recognition and appropriate treatment can, however, greatly reduce mortality (22, 24). Unfortunately, identification of the causative species at the genus level may require detection of diagnostic amino acids, sugars, fatty acids, mycolic acids, phospholipids, and menaquinones; this may be beyond the capabilities of most laboratories. High-performance liquid chromatography, for example, has been shown to be able to identify the nocardioforms to the species level by mycolic acid analysis, greatly reducing the time required for diagnosis (6, 11). Definitive diagnosis by using current clinical methods requires about 2 weeks because these methods rely on substrate decompositions and fermentation reactions (13, 14). Serological studies using culture filtrate antigens have demonstrated several *N. asteroides* serotypes and a species-specific antigen for *N. otitidiscaviarum* (23, 25). Serodiagnosis using the immunodominant proteins found in culture filtrates as antigens has been attempted. The immunodominant proteins were found to react with sera from patients with systemic infections but were not species specific (1, 28; A. M. Sugar, personal communication).

The purpose of the present study was to isolate DNA probes for the rapid and specific identification of *N. asteroides*. The advantage of DNA probes over even the high-performance liquid chromatography technology is that, when combined with target amplification or polymerase chain reaction, the need to culture these slowly growing organisms may be eliminated. DNA probes are now available for use in the identification of several *Mycobacterium* species (8, 17, 26). Because the nocardiae may produce infections in both healthy and compromised hosts that are clinically indistinguishable from those caused by the mycobacteria, an *N. asteroides* probe could be very useful.

MATERIALS AND METHODS

Bacterial strains. A reference collection of nocardioform bacteria was screened with DNA clones obtained from a genomic library of *N. asteroides* GUH-2. This strain we obtained from Blaine Beaman, University of California School of Medicine, Davis. It was selected for construction of the first library because it had previously been used in several studies on nocardial pathogenesis (2, 4, 5). All subsequent libraries were generated from selected reference collection *N. asteroides* strains that failed to hybridize with any of the GUH-2 clones tested. The reference collection comprised 28 strains of *N. asteroides* and 22 related species. The isolates were obtained from a variety of sources, including the Centers for Disease Control in Atlanta, Ga.; the American Type Culture Collection, Rockville, Md. (ATCC 19247, ATCC 4277, ATCC 184, ATCC 1368, ATCC 14887, ATCC 19370, ATCC 15905, ATCC 19369, and ATCC 19296); the University of California, Davis, Medical Center, Davis; the Medical College of Georgia, Augusta; the Eisenhower Army Medical Center, Fort Gordon, Ga.; Florida Institute of Technology, Melbourne; the National Institutes of Health, Bethesda, Md.; Hospital Gea Gonzales, Mexico City, Mexico; and the University of Los Andes Hospital, Merida, Venezuela.

Strain cultivation. Mycobacteria were maintained on Middlebrook 7H11 medium (Difco Laboratories, Detroit, Mich.) and cultured in TB broth base (Difco) with 5% serum albumin fraction V and 7.5% glucose. The cultures were incubated for 3 days without shaking at 37°C and then in a rotary shaker at 180 rpm for an additional 5 days to 3 weeks, depending upon the growth rate of the strain. The nocardiae and other *Actinomycetales* were cultured on brain heart infusion medium (Difco). *Rhodococcus* spp. were incubated at 30°C in a shaking incubator for 24 to 48 h and the nocardiae were cultivated for 72 h to 1 week at 37°C.

DNA isolation. For DNA extraction, most of the strains had to be cultured in media that increased their susceptibility to lysozyme. Consequently, those strains that were selected for construction of genomic libraries were cultured in brain heart infusion broth containing 10% sucrose, with the addition of glycine (2 to 5%) 48 h before harvest. For the other reference strains, 50 μg of ampicillin per ml was used in place of glycine. Ampicillin treatment proved to be less effective than the glycine treatment but still provided sufficient yields of DNA for testing the probes and eliminated the need for determining the appropriate concentration of glycine needed for each strain. Mycobacterial strains were also treated with glycine before lysis (20). Treated cells were centrifuged, washed with phosphate-buffered saline containing 10% sucrose (pH 7.2), and resuspended in 3 ml of the

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same solution per 50 ml of original culture medium. Dry lysozyme (10 mg) and 0.1 ml of aqueous EDTA (0.5 M) were added to the suspension, and the mixture was incubated for 60 min at 37°C. Cells were lysed by the addition of 1.0 ml of a 10% dilution of sodium dodecyl sulfate (SDS) and heating for 10 min at 68°C. Following lysis, 2 volumes of 10 mM Tris-HCl–1 mM EDTA (pH 8.0) were added to the lysate. Next CsCl (1.0 g/ml) and 0.5 ml of a 10-mg/ml stock solution of ethidium bromide were added, and the mixture was stored at room temperature in the dark for at least 2 h. The precipitated debris was removed by centrifugation at 2,000 × *g* for 20 min at 4°C. The clear fluid was removed from the bottom of the tubes, and the DNA was centrifuged to equilibrium in a Beckman 50 Ti rotor at 45,000 rpm for 48 h at 20°C. Standard DNA purification methods were then followed (19).

Production of libraries. DNA from selected strains was partially digested with *Bam*HI restriction endonuclease (Bethesda Research Laboratories, Gaithersburg, Md.), and fragments ranging from 9 to 15 kilobase pairs were isolated on strips of DEAE-cellulose paper by agarose gel electrophoresis (9, 19). The purified fragments were then ligated to bacteriophage lambda EMBL3 arms (Promega Corporation, Madison, Wis.) as specified by the manufacturer. This vector can accommodate 9- to 23-kilobase-pair fragments (15, 16). The in vitro packaging of the recombinant molecules was accomplished by use of Packagene (Promega). Selection for recombinant constructions is based on the Spi^+ phenotype in restrictive host *Escherichia coli* NM539 (12, 19).

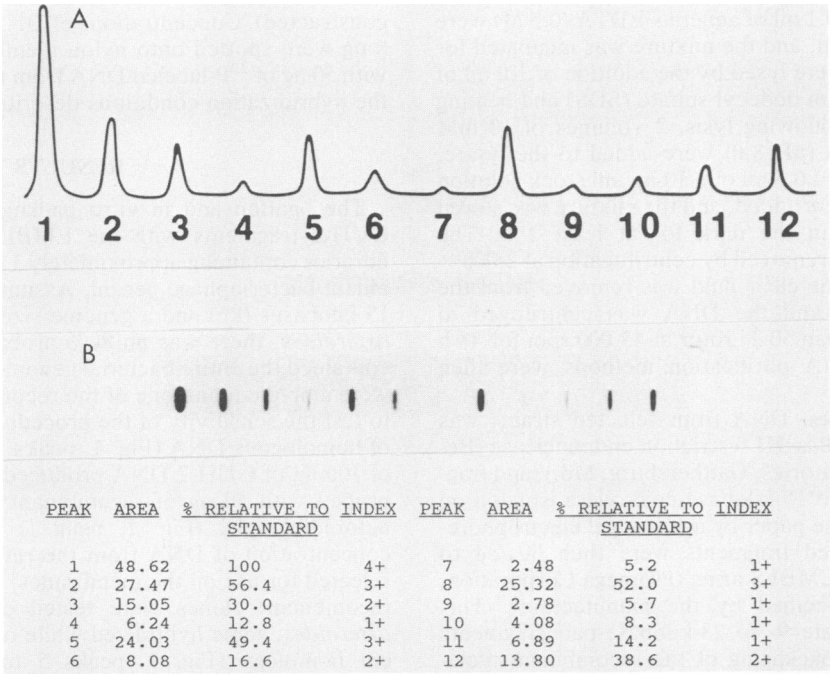
Preparation of phage DNA, labeled probes, and hybridization membranes. Phage plaques randomly selected from the libraries were amplified in small-scale liquid cultures by the method of Leder et al. (18). After the DNA was isolated from the phage (19), 50 ng was labeled with [^{32}P]dCTP (ICN Radiochemicals, Irvine, Calif.) by use of an oligonucleotide labeling kit (Pharmacia, Inc., Piscataway, N.J.) to an average specific activity of 10^8 cpm/μg of DNA (10). Hybridization was performed by using prewetted nylon membranes (Zetaprobe; Bio-Rad Laboratories, Richmond, Calif.) and hybri-slot or dot blot manifolds (Bethesda Research Laboratories). DNA (0.1 μg, unless otherwise noted) was applied in 20-μl volumes with constant vacuum. The DNA was denatured by rinsing wells with 0.5 ml of 0.4 M sodium hydroxide and by applying vacuum until wells were dry. Membranes were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), air dried, and then vacuum dried at 80°C for 2 h. The membranes were stored in sealed bags until hybridized (19).

Hybridizations and standardization of procedures. Membranes were prehybridized for 6 h at 60°C in a solution consisting of 6× SSPE (saline, sodium citrate, phosphate buffer, EDTA), 1.0% SDS, 0.5% Blotto (nonfat dry milk), and 0.5 mg of denatured salmon sperm DNA per ml. Membranes were hybridized for 24 h at 68°C with denatured labeled probe DNA (10^6 cpm). Following hybridization, membranes were washed stringently in 0.1× SSC–0.5% SDS at 68°C for 30 min to remove unhybridized probe. The membranes were then overlaid onto Kodak X-omatic film and an intensifying screen (Cronex Lightning-Plus; Du Pont) and exposed for 4 h at −80°C. After the film was developed, densitometric scans of the autoradiograms were performed on an autoscanner (Helena Laboratory, Beaumont, Tex.). Relative areas under each curve were determined by the electronic integrator of the autoscanner. The sensitivity of the procedure was determined by preparing membranes with serial two-fold dilutions of homologous *N. asteroides* GUH-2 DNA (the strain from which the first library was constructed). Concentrations of DNA ranging from 100 ng to 6 ng were spotted onto nylon membranes and then probed with 50 ng of ^{32}P-labeled DNA from the GUH-2 clones under the hybridization conditions described above.

RESULTS

The ligation and in vitro packaging of *Bam*HI-digested GUH-2 fragments with the EMBL3 vector arms yielded libraries containing approximately 1.45×10^4 PFU of recombinant bacteriophage per ml. Assuming an average insert of 15 kilobases (kb) and a genome size of 4.6×10^3 kb for *N. asteroides*, there was an 80% probability that the libraries contained the entire bacterial genome (7). Individual plaques were amplified, and one of the recombinant phage was used to test the sensitivity of the procedures by probing dilutions of homologous DNA (Fig. 1, peaks 1 to 4). A concentration of 100 ng of GUH-2 DNA produced an intense signal when probed with 50 ng of recombinant DNA following 4 h of autoradiography (Fig. 1, peak 1) and was therefore the concentration of DNA from the reference strains that was selected for use on the membranes. When the same GUH-2 recombinant clones were tested on other strains of *N. asteroides*, some hybridized while others showed no apparent homology (Fig. 1, peaks 5 to 12). In addition, the intensities of the hybridization reactions were greater with some strains than with others. Therefore, as a means of quantitating the data, a comparison was made between the relative intensity of the hybridized DNA from a given reference strain and that of a dilution of homologous DNA (GUH-2) which gave a comparable intensity. The percent area of each peak was calculated relative to the area of the 100-ng concentration of homologous DNA. A hybridization scale was developed by which an assigned index value could be used to score the intensity of the hybridization reactions. As can be seen on the scale (Table 1), an index value of 4+ correlates to the hybridization intensity equivalent to homologous GUH-2 DNA at a 100-ng concentration and a mean area relative to the homologous 100-ng standard of 92% or more. An index value of 3+ correlates with an intensity of a twofold DNA dilution and 50% of the relative area of the standard peak. An index value of 2+ correlates to a fourfold dilution of homologous DNA and represents 25% of the relative area of the standard peak. A 1+ index value represents a 10-fold dilution and 10% of the relative area of the standard peak. An index of 0 correlates with no reaction. For practical reasons, reactions giving index values of 2+ or greater were considered positive reactions because 1+ reactions were weak and difficult to interpret.

The hybridization reactions of 10 representative GUH-2 clones are provided in Table 2. The figures represent the percent hybridization to the reference strains at an index ranging from 2+ to 4+. Forty additional GUH-2 clones were also tested; however, they all showed hybridization patterns similar to that of one of the representative clones. Most of the clones tested showed cross-reactivity with strains of *N. brasiliensis* and *N. otitidiscaviarum* but were nonreactive when hybridized with other related genera. Only three clones (clones 3, 22, and 26) failed to cross-hybridize with other nocardiae. Clones 22 and 26 hybridized with the same strains, which left the combination of clones 3 and 22 as the only species-specific probes. Their combined reactivity detected 31% of the *N. asteroides* reference strains. There were several strains of *N. asteroides* that failed to hybridize with any of the 50 GUH-2 clones tested. These nonreactive



PEAK	AREA	% RELATIVE TO STANDARD	INDEX	PEAK	AREA	% RELATIVE TO STANDARD	INDEX
1	48.62	100	4+	7	2.48	5.2	1+
2	27.47	56.4	3+	8	25.32	52.0	3+
3	15.05	30.0	2+	9	2.78	5.7	1+
4	6.24	12.8	1+	10	4.08	8.3	1+
5	24.03	49.4	3+	11	6.89	14.2	1+
6	8.08	16.6	2+	12	13.80	38.6	2+

FIG. 1. Hybridization reactions between homologous DNA and DNA from some *N. asteroides* reference strains. (A) Densitometric scan of autoradiogram. (B) Autoradiogram. Peaks 1 to 4 represent homologous GUH-2 DNA dilutions from 100 ng to 12.5 ng. Peaks 5 to 12 are 100 ng of DNA from some of the *N. asteroides* reference strains. The membranes were probed with 50 ng of ^{32}P-labeled GUH-2 clone DNA with a specific activity of 10^8 cpm/μg of DNA. Membranes were exposed to film for 4 h at −80°C before development. The areas for each sample were densitometrically determined, and the percent area relative to the 100-ng sample of homologous DNA was determined and correlated to an appropriate hybridization index.

strains were used to generate EMBL3 libraries, and their clones in turn were tested for strain specificity (Table 2). Thirty of the nonreactive clones (five from each strain) were used to screen a collection of 17 strains of *N. asteroides* that gave poor or no hybridization signals with the GUH-2 clones. Five clones were identified (29-2, 2B-2, 26-4, 05-2, and A-1) that hybridized to previously nonreactive strains without cross-reacting with related species or genera (Table 2). The two GUH-2 clones and four clones from the other *N. asteroides* strains were then pooled, and the resulting mixture was used to probe membranes containing dot blots of 100 ng of reference strain DNA. Positive hybridization signals occurred only with *N. asteroides* strains (Table 2 [Pool]), and no cross-reactivity was observed with related organisms in the reference collection.

DISCUSSION

Testing of 50 recombinant clones from the GUH-2 strain of *N. asteroides* produced 2 clones that were found to be suitable for use as species-specific probes. However, they recognized only 31% of the *N. asteroides* strains with a hybridization intensity of 2+ or greater. While many more clones would have had to be tested to ensure a reasonable sampling of the GUH-2 chromosome, a group of consistently nonreactive or weakly reactive (1+) *N. asteroides* strains was recognized. The continued screening of the GUH-2 clones was discontinued because it had been anticipated that more than one strain of *N. asteroides* would have to be used to develop a specific diagnostic probe. This assumption was based on numerical taxonomy and DNA homology studies that clearly demonstrated the heterogeneous nature of this taxon (21, 27). Schaal and his colleagues recognized three subgroups of *N. asteroides* (A, B, and C) based on a cluster analysis on 128 strains and 139 characteristics (27). Because DNA homology data tend to support their observations, some systematists believe that the subgroups merit species status. Subgroup B is reported to include some strains designated *Nocardia farcinica*, a pathogenic species that is not usually distinguished from *N. asteroides* by routine clinical laboratory tests. There is, however, renewed interest in *N. farcinica* following reports that it shows unique drug susceptibility patterns in that it is resistant to broad-spectrum cephalosporins and the prognosis of its infections may differ from that of infections caused by the other nocardiae (29, 30; R. J. Wallace, personal communication). Reference strain 2B (clone 2B2) is representative of subgroup B and is currently being tested for its reactivity to strains designated *N. farcinica*. When clones were analyzed electrophoretically after *Bam*HI digestion, the cloned insert for probe A-1 was 16.2 kb. The other clones were all found to contain partial genomic digests. Clone 29-2 contained 6.8-,

TABLE 1. Hybridization index scale

Index	Intensity equivalent to homologous GUH-2 DNA[a]	% Area relative to homologous DNA (mean ± SD)[b]
4+	Equivalent	92.0 ± 6.4
3+	Twofold dilution	54.0 ± 6.6
2+	Fourfold dilution	29.0 ± 7.3
1+	10-fold dilution	10.0 ± 4.8
0	No homology	

[a] The index values are correlated to intensities that are equivalent to various dilutions of homologous GUH-2 DNA.

[b] The relative percent area was determined densitometrically and is represented by twofold dilutions of a 100-ng sample of DNA.

TABLE 2. Hybridizations between clones derived from strains of *N. asteroides* with *Nocardia* spp. and other related *Actinomycetales*

Taxon (no. of isolates)	% Reactive strains at an intensity of 2+ or greater[a]																		
	GUH-2 clones										Nonreactive clones								
	1	2	3	5	10	22	23	24	26	27	29-1	29-2	05-2	2B2	26-4	A-1	A-2	2B-2	Pool[b]
N. asteroides (28)	45	31	20	58	48	24	34	49	14	28	77	65	82	59	83	53	41	35	100
N. brasiliensis (3)	33	33	0	100	100	0	66	66	0	33	33	0	0	0	0	0	33	0	0
N. otitidiscaviarum (3)	0	0	0	100	33	0	33	66	0	0	33	0	0	0	0	0	33	0	0
Actinomadura sp. (3)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mycobacterium sp. (8)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhodococcus sp. (5)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] GUH-2 clones were derived from *N. asteroides* GUH-2; nonreactive clones were obtained from strains which did not hybridize with any of the GUH-2 clones tested (see text).

[b] Combination of clones GUH-3, GUH-22, 29-2, 26-4, A-1, and 2B2.

4.8-, 4.4-, and 3.6-kb fragments. Clone 26-4 had 6.6-, 4.6-, and 3.6-kb inserts, and clone 2B2 consisted of 5.4- and 4.3-kb fragments, while clones GUH-3 and GUH-22 had doublets of 5.4 and 4.3 kb, respectively. The large size of the cloned fragments may account for the observed variations in intensity of the hybridization reactions; i.e., weak or 1+ reactions may reflect hybridizations of only very short regions of the probes with the reference strains. For clinical application and for the use of nonradiolabeled probes, it may be desirable to eliminate these weak hybridization reactions. This could be done by subcloning the individual fragments or by the reduction of the size of the clones. Alternatively, subtractive hybridization could be used (31). The latter technique requires passage of a mixture of nick-translated probe and reference strain DNA over a Sepharose-acid-agarose column to bind hybridized fragments. The probe fragments passing through the column are recognized by using alternative label methods (avidin-biotin, etc.) and should be free of any remaining hybridization activity. Following these refinements, primers for target amplification could be produced which, in turn, would greatly enhance the utility of the probes in direct analysis of contaminated low-yield specimens, such as sputum.

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